Analysis of Atrazine Residues in Food by an Enzyme Immunoassay†

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The application and validation of a highly sensitive and relatively selective enzyme immunoassay (EIA) for the analysis of atrazine residues in food is described. The assay is performed on microtiter plates. The range of detection for atrazine analysis lies between approximately 1 ng/L and 10 μg/L. Various foods in liquid form such as milk, fruit juices, and vegetable juices and in solid form such as corn were analyzed with the EIA in their natural states and after the samples were spiked with defined amounts of atrazine. The solid food samples had to be extracted before EIA analysis. However, the assay did not require concentration or cleanup steps for any of the liquid foodstuffs analyzed. A comparison with HPLC measurements was made with the extracted food samples. A close correspondence was found between the results of the EIA and HPLC measurements, although the extraction yield proved to be relatively low (between 20 and 50%). In the case of the liquid forms, highly accurate measurements of the actual concentrations were obtained with the EIA.

INTRODUCTION

Worldwide, several groups have developed enzyme immunoassays (EIA) for the analysis of *s*-triazines with polyclonal and monoclonal antibodies (Bushway et al., 1988; Schlaeppi et al., 1989; Wittmann and Hock, 1989, 1990; Dunbar et al., 1990; Goodrow et al., 1990; Thurman et al., 1990; Harrison et al., 1991). The main application for these assays is in the field of water analysis. The field application of atrazine was permitted until the end of 1990 in the Federal Republic of Germany. Since, after the aromatic carboxylic acids, the *s*-triazines (and within that group primarily atrazine till the end of 1990) were the most widely used herbicides (Kraus, 1989), it seems appropriate to determine atrazine residues in foods, too, especially considering the persistency of atrazine and the fact that atrazine is still allowed and frequently used in other countries.

Atrazine is used as a pre- or post-emergent herbicide and is selectively directed against weeds and couch grass (quack grass) in cultures of corn, asparagus, and stone fruit and in viticulture. It is also applied as a total herbicide on roads and agricultural or uncultivated areas as well as in combination with other pesticides and growth regulators. The uptake by the plants occurs primarily via the roots and partly through the leaves. The *s*-triazines inhibit photosynthesis by blocking the electron transfer between photosystems II and I after binding to the Q_B protein (*The Pesticide Manual*, 1987).

Atrazine in plants (e.g., corn and fruit) and consequently in many foods is mainly analyzed by gas chromatography (GC) or high-pressure liquid chromatography (HPLC). Both methods require extraction, i.e., sample preparation, and in some cases enrichment steps prior to determination. Therefore, rather expensive and sophisticated technical equipment is needed. Immunoassays, on the other hand, have the advantage of being less expensive and usually more rapid and sensitive, while the disadvantages commonly include cross-reactivities of the applied antibody with other, related compounds as well as matrix effects.

Because of the extensive application (until the end of 1990 in the Federal Republic of Germany), limited toxicological data over a long period, and great persistency of atrazine, there is a need for a routine monitoring of foods in addition to water and soil for atrazine residues.

An *s*-atrazine EIA with a measuring range of 0.5–10 μg/L for atrazine is described by Bushway et al. (1989) for atrazine analysis in foods. However, this assay was limited in its application because an original matrix without atrazine was required to prepare the standard series.

The aim of this paper was to test an enzyme immunoassay recently introduced and evaluated for the determination of atrazine in environmental water samples, soil and plant extracts, and sediment (Wittmann and Hock, 1989, 1990) for its suitability for monitoring atrazine residues in food. This was facilitated by the absence of propazine (a cross-reacting substance for the antibody used in this assay) which, with few exceptions, has not been used in Europe. It is shown that with this atrazine EIA liquid foods such as milk, fruit juices, and vegetable juices could be directly measured. There was no need for any cleanup. The solid food extracts did not require a concentration step before measurement with the EIA.

MATERIALS AND METHODS

1. **Materials.** *1.1. Chemicals.* The polyclonal atrazine antibody C193 (blood collection on May 17, 1989) and the horseradish peroxidase hapten tracer used in the assay were prepared as described earlier (Wittmann and Hock, 1989). The triazine standards were provided by Riedel de Haen, Seelze, and Ciba Geigy, Basel. In addition, the following reagents were used: tetramethylbenzidine (Riedel de Haen), hydrogen peroxide, 30% (Merck, Darmstadt), and ethanol absolute, p.a. (Merck). All other chemicals used were of analytical grade.

1.2. Buffers and solutions used included the following: (1) carbonate buffer, 50 mmol/L, pH 9.6, for coating; (2) phosphate-buffered saline (PBS), 40 mmol/L, pH 7.2 (containing 8.5 g of NaCl/L) for the preparation of standards and the peroxidase tracer; (3) PBS washing buffer, 4 mmol/L, p 7.2 (containing 0.85 g of NaCl/L and 0.5 mL of Tween 20/L) for washing and microtiter plates; (4) substrate buffer for peroxidase—0.1 M sodium acetate buffer, pH adjusted to 5.5 by adding 1 M citric acid; (5) substrate for peroxidase—400 μL of tetramethylbenzidine (TMB; 6 mg of TMB was dissolved in 1 mL of dimethyl sulfoxide) plus 100 μL of 1% (v/v) H_2O_2 filled up to 25 mL with substrate buffer; (6) stopping reagent—H_2SO_4, 2 mol/L.

1.3. Preparation of Atrazine Standards. Atrazine (5 mg) was dissolved in 50 mL of absolute ethanol with the aid of an ultrasonic

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bath (20 min). Starting with this solution, a stock solution was prepared consisting of 1 mg/L atrazine (=excess, i.e., the concentration that reduces the signal of its minimum level). A standard series was prepared by making several dilutions of the stock solution containing the following atrazine concentrations: 0.01, 0.03, 0.1, 0.3, 1, and 10 μg/L. The stock solution as well as the standard series was made up either in 40 mmol of PBS buffer, pH 7.2, or in distilled water.

1.4. Equipment. The laboratory equipment used comprised an EIA photometer [SLT Easy Reader EAR 400 (SLT, Gröding/Salzburg, Austria)]; a microtiter plate washer [SLT Easy Washer EAW 8112 (SLT)]; an ultrasonic bath (Sonorex Bandelin); and a HPLC apparatus [UVIKON 720 LC (Kontron, Eching, FRG)] including a HPLC column [ODS 5-μm Spherisorb (Kontron)].

1.5. Further materials used included the following: 96-well microtiter plates, type F-form, high binding capacity, No. 655061 (Greiner Labortechnik, D-72636 Frickenhausen, FRG); TLC plates silica gel 60, F_{254} (Merck); Sep-Pak C_{18} columns (Millipore, Eschborn).

2. Methods. Details of antibody production and enzyme tracer synthesis for the development of an atrazine enzyme immunoassay have been described elsewhere (Wittmann and Hock, 1989).

The EIA was performed in microtiter plates at room temperature. The protocol for the optimized assay includes the following steps:

2.1. Coating. Three hundred microliters of an antiserum dilution of 1:50000 in carbonate buffer, pH 9.6, is incubated at 4 °C overnight or at 37 °C for 4 h.

2.2. Washing. Three washes are performed with 300 μL of PBS washing buffer, pH 7.2, by using the microtiter plate washer. The buffer is removed after the last step.

2.3. Immunoreaction. Two hundred microliters of standard or sample is added, followed by 50 μL of an enzyme tracer dilution of 1:50000 in PBS buffer, pH 7.2. After the plate is agitated on a horizontal shaker for 1 min, it is incubated for 1 h at room temperature.

2.4. Washing is done three times as before.

2.5. Enzyme Reaction. Two hundred microliters of substrate is added. The enzyme reaction is stopped after 10 min with 100 μL of 2 mol/L H_2SO_4. Then the absorption is read at 450 nm. Each determination is performed in quadruplicate. An atrazine standard series was run on each microtiter plate.

The absorptions (A) are converted to % B/B_0 values according to the formula

$$\% B/B_0 = (A - A_{excess})/(A_{control} - A_{excess}) \times 100$$

and finally transformed to logit values for linearization of the calibration curve according to the formula

$$\text{logit}(\% B/B_0) = \ln[(\% B/B_0)/(100 - \% B/B_0)]$$

The evaluation of the cross-reactivities was performed as described previously (Wittmann and Hock, 1989).

When necessary, the samples were adjusted to a pH between 7.0 and 7.5 [i.e., usually one part PBS buffer, pH 7.2, plus nine parts sample (v/v)] for the measurement of the liquid foods and the extracted solid foods. When the samples contained organic solvents, they were diluted to a final concentration of 1% of the organic solvent. Higher solvent concentrations can disturb the test.

The liquid foods (milk, fruit juices, and vegetable juices were chosen as representative examples) were directly measured with the EIA. First, a standard series was made up in the respective food after pH adjustment as described above and compared with an atrazine standard series prepared in distilled water. The food samples were spiked with atrazine in concentrations of 0.05, 0.1, and 1 μg/L. As a standard series for calculating the atrazine contents of the prepared food samples, the one prepared in the respective food and the other made up with distilled water were compared in the EIA.

For HPLC analysis and an accompanying determination with the EIA UHT milk (*H-Vollmilch*, fat content 1.5%), orange-juice drink (*Orangennektar*), and canned corn were chosen as representative samples. The extraction method was chosen because this procedure was already established for atrazine analysis in water plants in the same laboratory (Prof. Dr. W. Huber, Institute of Botany and Landscape Architecture, TU München at Weihenstephan) where the HPLC analyses were performed. In addition, the isolation procedure offered the possibility to use a uniform extraction for all foods analyzed. Therefore, the foods chosen as representative examples were extracted according to a method modified from that of Huber (unpublished procedures).

The milk and the juice were treated in an analogous manner. Three different variations were always prepared from each sample: (a) in the natural state, (b) spiked with 1.5 μg of atrazine/100 mL of the sample, and (c) fortified with 3 μg of atrazine/100 mL of sample. One hundred milliliters of the sample was shaken out twice with 100 mL of ethyl acetate each time and then vaporized to dryness in a rotary evaporator. The residue was then dissolved in 5 mL of methanol and made up to 250 mL with distilled water. This solution was applied on a Sep-Pak C_{18} column which was washed before with 2 mL of acetone. Elution was performed with 2 mL of acetone. The acetone extract was concentrated with a stream of nitrogen gas and dissolved in 200 μL of methanol. One hundred microliters of the methanolic extract was then put on a TLC plate (silica gel 60, F_{254}). Atrazine was separated from other sample ingredients in the solvent system 85/15 (v/v) toluene/acetone. As a reference substance 500 μL of a standard solution containing 15 mg of atrazine/50 mL of methanol was put on the TLC plate and separated in the same manner. The substances were detected under a UV lamp (254 nm). At the height of the reference substance the zones (stripes) were generously scraped off of the silica gel and subsequently extracted twice with 1.5 mL of ethyl acetate each. The extract was shaken for 1 min and then centrifuged for 15 min in an Eppendorf centrifuge at 12 000 rpm. The supernatants were combined and vaporized to dryness under nitrogen. The residue was dissolved in 200 μL of methanol and stored in the refrigerator until the measurement of the methanolic extracts by HPLC. For the determination with the EIA the methanolic extracts were diluted with distilled water to 10 mL.

Canned corn was used as a representative example of a solid food. Three samples were prepared: (a) in the natural state, (b) spiked with atrazine in a concentration of 3 μg/10 g of sample, and (c) fortified with 6 μg of atrazine/10 g of sample. Ten grams of the sample was suspended in 50 mL of methanol and minced for 10 min with the aid of a Starmix on setting 3. The mixture was rinsed with 50 mL of methanol before the mincing procedure was repeated. The extracts were dried with a rotary evaporator. Then the residues were dissolved in 5 mL of methanol and filled up to 250 mL with distilled water. Each sample was then applied to a Sep-Pak C_{18} column and treated in the same manner as described for the milk and the juice after Sep-Pak C_{18} column cleanup.

The following HPLC conditions were employed: column, ODS 5-μm Spherisorb; carrier (mobile phase), 80/20 (v/v) methanol/water; flow rate, 10 mL/min; UV detector, 230 nm.

Twenty microliters of the methanolic extract was injected into the column. With the aid of an external (and, for comparison, the internal) atrazine standard, the atrazine concentrations of the injected sample extracts were calculated by comparing the integrated peak areas. Parallel to the HPLC measurements the aqueous food extracts were analyzed with the EIA.

RESULTS

Analyses of plant extracts, which were performed with a recently introduced atrazine EIA by the group of Prof. Dr. W. Huber (Institute of Landscape Architecture and Botany, TU München at Weihenstephan) in aquatic model ecosystems, yielded fairly accurate results which were in accordance with HPLC results [cf. Wittmann and Hock (1990)]. Therefore, the question presents itself whether foods, especially if they are derived from plants, or their extracts could also be analyzed with the EIA for potential atrazine contamination. The newly developed atrazine EIA covered a measuring range from 1 ng/L to 10 μg/L with a coefficient of variation (CV) of 4%. At first, liquid food samples, mainly milk and fruit and vegetable juices,

Table I. Direct Analysis of Liquid Foods with the Atrazine EIA[a]

sample	atrazine concn added, μg/L	atrazine concn determined with the EIA, μg/L ± SD	deviation, %
milk		<0.001	
milk	0.05	0.06 ± 0.002	14
milk	0.10	0.12 ± 0.004	20
milk	1.00	1.02 ± 0.040	1.7
apple juice		<0.001	
applie juice	0.05	0.05 ± 0.001	4
apple juice	0.10	0.10 ± 0.004	4
apple juice	1.00	1.02 ± 0.041	2

[a] As representative samples a milk (fat content 3.5%) and an apple-juice drink are shown. The samples were analyzed first for a possible contamination with atrazine in the natural state, then spiked with atrazine in concentrations of 0.05, 0.10, and 1.00 μg/L, and measured in the EIA. The atrazine concentration of the samples could be calculated with the atrazine standard series prepared in distilled water.

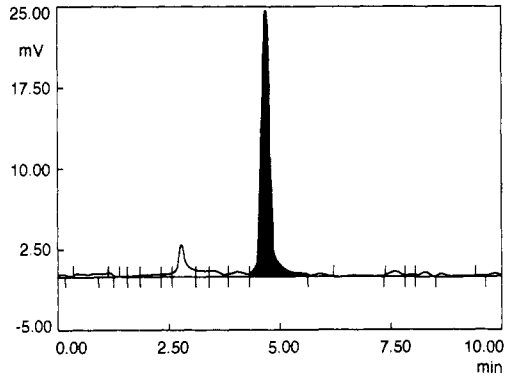

Figure 1. HPLC chromatogram of the atrazine standard. The peak area (4.98) at the retention time of 4.63 min corresponds to 120 ng of atrazine.

were tested directly in the EIA. The samples were spiked with three different atrazine concentrations and measured in the EIA to determine the recovery rates for atrazine. The data were calculated on the basis of a standard series of atrazine prepared in the respective food or in distilled water. Table I shows the results for representative samples of apple juice and milk. The atrazine could be totally recovered with a high reproducibility. Most importantly, the samples could be measured directly in the EIA without any cleanup required. Moreover, no matrix effects were observed. An atrazine standard series prepared in distilled water could be used as a calibration curve. Both samples turned out to be free of atrazine. The CV for the EIA determination amounted to 4% on the average.

In a next step extracts of a UHT milk (*H-Vollmilch*), an orange-juice drink (*Orangennektar*), and a canned corn sample were prepared. The samples were taken in their natural states and fortified with defined amounts of atrazine. These food extracts were then determined in parallel by HPLC and with the EIA for a direct comparison of the two analytical methods. Figure 1 shows the HPLC chromatogram of the atrazine standard. The atrazine peak appeared at a retention time of 4.63 min. The HPLC chromatogram of the extract of the orange-juice sample in the natural state is known as a representative example in Figure 2. A peak appeared at a retention time of 4.64 min. It was classified as atrazine because the analyte peak was enhanced when the extracts were spiked. In addition, the atrazine peak was confirmed by GC analysis using a nitrogen–phosphorus-selective detector.

EIA analysis confirmed the identity of the substance as atrazine. The antibody used for this assay reacted

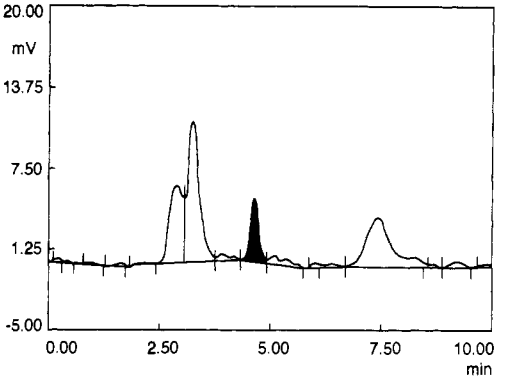

Figure 2. HPLC chromatogram of the methanolic extract of an orange-juice sample in the natural state. The peak area (1.08) at the retention time of 4.64 min was identified as 26 ng of atrazine (=5.2 μg/L).

Table II. Comparison of EIA and HPLC Data from the Measurement of Food Extracts[a]

sample	atrazine concn of the spiked sample	atrazine concn determined by HPLC	atrazine amount determined with the EIA	recovery rate of HPLC analysis, %
milk		<0.001 μg/L	<0.001 μg/L	
milk	15 μg/L	3.06 μg/L	2.96 μg/L	20.4
milk	30 μg/L	5.19 μg/L	4.92 μg/L	17.3
orange juice		5.2 μg/L	4.9 μg/L	
orange juice	15 μg/L	4.58 μg/L	4.82 μg/L	30.5
orange juice	30 μg/L	9.9 μg/L	10.1 μg/L	33
canned corn		0.05 mg/kg	0.05 mg/kg	
canned corn	3 μg/10 g	0.16 mg/kg	0.16 mg/kg	53
canned corn	6 μg/10 g	0.27 mg/kg	0.29 mg/kg	44.9

[a] As representative samples a UHT milk (fat content 1.5%), an orange-juice drink, and canned corn were chosen. Extracts were prepared of each sample in the natural state and after the sample was fortfied with defined atrazine amounts. The methanolic extracts were analyzed with HPLC. If the chromatogram exhibited a peak after a retention time of 4.63 min, the atrazine contents of the extracts were calculated by comparing the integrated peak areas of the sample with the atrazine standard. For EIA measurement aqueous extracts were taken and each sample was analyzed in quadruplicate.

selectively with atrazine (100%) and propazine (195%). Simazine was detected with a considerably lower affinity (20%). Because propazine has not been used in Europe with the exception of Belgium and the Netherlands, the EIA could be applied as a highly sensitive screening test for atrazine. Table II shows the results of EIA and HPLC measurements for the prepared food extracts. It can be recognized that there is a good correlation between HPLC and EIA results for the orange-juice drink in the natural state. However, if the sample contained propazine or simazine, the amounts detected by the EIA on the basis of an atrazine standard series would have been higher for propazine and considerably lower for simazine in the case of propazine or simazine contamination, respectively, because of their different cross-reactivities.

For comparison, the orange-juice drink was analyzed directly in the EIA without any extraction prior to the analysis. The result was 15.5 μg/L atrazine, which correlated favorably to the extrapolated amount of 16 μg/L atrazine for the original sample by HPLC analysis. An analysis of the extract by HPLC and EIA yielded a good correspondence between the two methods. However, the recovery rates turned out to be rather low due to considerable losses of atrazine during extraction. The extraction yield was reproducible for a defined foodstuff, meaning the recoveries depended on the food analyzed. In our case the mean recovery rates were determined with

the spiked food extracts and allowed an estimation of the real atrazine contents of the sample. For milk, a mean recovery rate of 19% was found, for orange juice, 32%, and for the canned corn sample, 49%, on the basis of food samples spiked with atrazine. In addition, the recovery rates for the orange juice and the canned corn sample were calculated by adding an internal terbuthylazine standard (data not shown) to the samples in the natural state, yielding recoveries of 35% for the orange juice and 50% for the canned corn sample, thus confirming the previous results.

It was confirmed by HPLC and EIA data that the original canned corn and the orange-juice samples both exhibited contamination by atrazine. The real atrazine amounts were estimated to be 96 μg/kg canned corn and 16 μg/L orange juice according to the recovery rates calculated in Table II.

DISCUSSION

It was shown that the atrazine EIA described in this paper can be applied to the analysis of foods. Liquid foods could be measured directly in the EIA. This means a considerable reduction in analysis time as compared to the HPLC method, combined with a recovery of nearly 100% in the EIA. No matrix effects were observed in this case. For this reason, the atrazine standard series could be prepared in distilled water; i.e., there was no need to return to the respective matrix. Direct analysis of liquid foods avoided losses from incomplete extraction. However, an extraction is needed for the analysis of solid foods. EIA analysis does not require enrichment steps. The results of HPLC and EIA measurements of food extracts showed a high correlation. We envision an important application of EIA in the confirmation of HPLC and GC analyses, especially with liquid foods as samples, and in its use as a screening method.

The isolation procedure for atrazine applied to the food samples is not recommended for food in general because the loss of atrazine during extraction was rather high. For milk, a mean recovery rate of 19% was found, for orange juice, 32%, and for the canned corn sample, 49%, on the basis of food samples spiked with atrazine. In addition, the recovery rates for the orange juice and the canned corn sample were calculated by adding an internal terbuthylazine standard (data not shown) to the samples in the natural state, yielding recoveries of 35% for the orange juice and 50% for the canned corn sample, thus confirming the previous results. An improvement in the extraction method with regard to a reduction in the number of extraction steps and an increase in atrazine recovery seems to be desirable in the future. Another possibility is to determine the reproducibility for each defined foodstuff and extrapolate the results to 100% as the analysis of the orange juice by HPLC and direct EIA analysis showed. For EIA determination it can be assumed that a considerable simplification of extraction might be possible because the extracts did not have to be of such high purity (quality) as for HPLC. An orange-juice sample and the canned corn sample exhibited atrazine contamination.

The pollution of the canned corn was no surprise because atrazine was frequently applied to corn fields. On the contrary, no treatment of orange cultures with atrazine has been described. It is suggested that the pollution results from the water and sugar which are allowed in orange-juice drinks (*Orangennektar*). The atrazine concentrations of both samples were below the maximum concentration for atrazine set forth in the ordinance for pesticides in food from October 16, 1989. This "Höchstmengenverordnung" regulates the maximum permissible concentration of pesticides in foods. A maximum atrazine amount of 10 mg/kg is allowed in fungi (mushrooms), 1 mg/kg in sweet corn, 0.5 mg/kg in corn, and 0.1 mg/kg in other plant foods. Spices, coffee, tea and products similar to tea, and oil seeds may contain up to 10 mg/kg. None of the assayed samples of canned corn and orange juice exhibited atrazine concentrations above the respective limit. In this context, an interesting aspect was the difference between the maximum concentration for pesticides in drinking water and the limit for pesticides in food, differing by a factor of 10^3–10^5. The limits of the drinking water ordinance should be understood as a quasi zero control to prevent an accumulation of pesticides in drinking water as early as possible. None of the limits are based upon toxicity levels. Many research groups are dealing with the toxicity, effect, and metabolism of *s*-triazines (Kunze, 1989; Lardier and Schiavon, 1989; Reed et al., 1990). According to Reed et al. (1990), the acute oral LD_{50} of atrazine in rats amounted to 5100 mg/kg of weight and in rabbits, 9300 mg/kg. For this reason atrazine is not classified as a highly toxic substance. Hamilton et al. (1988) described that a twofold application of 0.1 mg/L atrazine to microcosms (microenclosures) of a lake was sufficient to inhibit photosynthesis of the phytoplankton to a damaging extent. The green algae required a long phase of regeneration. Marchini et al. (1988) concluded that the triazines are more toxic to fish than to Daphnia. The triazines with thiomethyl groups reacted more toxically than the ones substituted with chlorine. Toxicity increased successively from the triazines substituted with a methoxy group through the ones with two chlorine atoms to the triazines with a thiomethyl. Infurna et al. (1988) reported toxic effects of atrazine but no teratological events in rabbits and rats. Animals as well as humans excrete atrazine within 24 h via the urine. Adams et al. (199) examined the metabolism of atrazine in rats, mice, goats, sheep, pigs, rabbits, and hens. Deethylatrazine and deisopropylatrazine were detected as phase I metabolites, the *S*-glutathion conjugates of atrazine, deethylatrazine, and deisopropylatrazine in phase II. The degradation exhibited different velocities depending upon the animal species. The application of the EIA for atrazine analysis in urine and in blood is conceivable. Considering the positive experiences with the EIA determination of liquid foods, a direct EIA analysis should be possible.

The results of the EIA determinations correlated to a high degree with HPLC data so that aside from the analysis of atrazine in environmental water samples, sediment, plant and soil extracts, and foods, other applications are conceivable, for example, the analysis of biological matrices such as blood and urine.

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